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Takada, Yoshikazu

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Review Article

Potential Role of Kringle-Integrin Interaction in Plasmin and uPA Actions (A Hypothesis)

Yoshikazu Takada

Department of Dermatology, and Biochemistry and Molecular Medicine, University of California Davis School of Medicine, Research III Suite 3300, 4645 Second Avenue, Sacramento, CA 95817, USA

Correspondence should be addressed to Yoshikazu Takada, ytakada@ucdavis.edu

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We previously showed that the kringle domains of plasmin and angiostatin, the N-terminal four kringles (K1–4) of plasminogen, directly bind to integrins. Angiostatin blocks tumor-mediated angiogenesis and has great therapeutic potential. Angiostatin binding to integrins may be related to the antiinflammatory action of angiostatin. We reported that plasmin induces signals through protease-activated receptor (PAR-1), and plasmin-integrin interaction may be required for enhancing plasmin concentration on the cell surface, and enhances its signaling function. Angiostatin binding to integrin does not seem to induce proliferative signals. One possible mechanism of angiostatin's inhibitory action is that angiostatin suppresses plasmin-induced PAR-1 activation by competing with plasmin for binding to integrins. Interestingly, plasminogen did not interact with $\alpha v \beta 3$, suggesting that the $\alpha v \beta 3$ -binding sites in the kringle domains of plasminogen are cryptic. The kringle domain of urokinase-type plasminogen activator (uPA) also binds to integrins. The uPA-integrin interaction enhances uPA concentrations on the cell surface and enhances plasminogen activation on the cell surface. It is likely that integrins bind to the kringle domain, and uPAR binds to the growth factor-like domain (GFD) of uPA simultaneously, making the uPAR-uPA-integrin ternary complex. We present a docking model of the ternary complex.

1. The Kringle Domains of Plasmin Interact with Integrins

The integrins are a superfamily of cell adhesion receptors that bind to extracellular matrix ligands, cell-surface ligands, and soluble ligands. They are transmembrane $\alpha \beta$ heterodimers and at least 18 α and eight β subunits are known in humans, generating 24 heterodimers [1]. The α and β subunits have distinct domain structures, with extracellular domains from each subunit contributing to the ligand-binding site of the heterodimer. The sequence arginine-glycine-aspartic acid (RGD) was identified as a general integrin-binding motif, but individual integrins are also specific for particular protein ligands. Immunologically important integrin ligands are the intercellular adhesion molecules (ICAMs), immunoglobulin superfamily members present on inflamed endothelium and antigen-presenting cells. On ligand binding, integrins transduce signals into the cell interior; they can also receive intracellular signals that regulate their ligand-binding affinity.

Angiostatin, a proteolytic fragment of plasminogen, contains either the first three or four kringle domains of plasminogen and is a potent inhibitor of tumor-induced angiogenesis in animal models [2, 3]. Angiostatin has promising therapeutic potential and is now in clinical trials. Plasminogen is first converted to the two-chain serine protease plasmin by cleavage of a single Arg561-Val562 peptide bond by urokinase-type plasminogen activator (uPA), and plasmin serves as both the substrate and enzyme for the generation of angiostatin [4]. Several other mechanisms have been proposed for the generation of angiostatin from the plasminogen molecule [5]. The antiangiogenic functions of plasminogen kringles have been extensively studied using recombinant plasminogen kringles and kringle fragments produced by elastolytic processing of native plasminogen. Smaller fragments of angiostatin display differential effects on the suppression of endothelial cell growth [6].

We found that bovine arterial endothelial (BAE) cells adhere to angiostatin in an integrin-dependent manner and

that integrins $\alpha v\beta 3$, $\alpha 9\beta 1$, and to a lesser extent $\alpha 4\beta 1$, specifically bind to angiostatin. $\alpha v\beta 3$ is a predominant receptor for angiostatin on BAE cells, since a function-blocking antibody to $\alpha v\beta 3$ effectively blocks adhesion of BAE cells to angiostatin, but an antibody to $\alpha 9\beta 1$ does not. ϵ -Aminocaproic acid, a Lys analogue, effectively blocks angiostatin binding to BAE cells, indicating that an unoccupied Lys-binding site of the kringles may be required for integrin binding. It is known that other plasminogen fragments containing three or five kringles (K1–3 or K1–5) have an antiangiogenic effect, but plasminogen itself does not. We found that K1–3 and K1–5 bind to $\alpha v\beta 3$, but plasminogen does not. These results suggest that the anti-angiogenic action of angiostatin may be mediated via interaction with $\alpha v\beta 3$. Angiostatin binding to $\alpha v\beta 3$ does not strongly induce stress-fiber formation, suggesting that angiostatin may prevent angiogenesis by perturbing the $\alpha v\beta 3$ -mediated signal transduction that may be necessary for angiogenesis [7].

Plasmin, the parent molecule of angiostatin and a major extracellular protease, induces platelet aggregation, migration of peripheral blood monocytes, and release of arachidonate and leukotriene from several cell types [8]. We found that plasmin specifically binds to $\alpha v\beta 3$ through the kringle domains and induces migration of endothelial cells. In contrast, angiostatin does not induce cell migration. Notably, angiostatin, anti- $\alpha v\beta 3$ antibodies, RGD-peptide, and a serine protease inhibitor effectively block plasmin-induced cell migration. These results suggest that plasmin-induced migration of endothelial cells requires $\alpha v\beta 3$ and the catalytic activity of plasmin and that this process is a potential target for the inhibitory activity of angiostatin [9].

We found that plasmin specifically interacts with integrin ($\alpha 9\beta 1$) and that plasmin induces migration of cells expressing recombinant $\alpha 9\beta 1$ ($\alpha 9$ -Chinese hamster ovary (CHO) cells). Migration was dependent on an interaction of the kringle domains of plasmin with $\alpha 9\beta 1$ as well as the catalytic activity of plasmin. Angiostatin, representing the kringle domains of plasmin, alone did not induce the migration of $\alpha 9$ -CHO cells, but simultaneous activation of the G protein-coupled protease-activated receptor (PAR)-1 with an agonist peptide induced the migration on angiostatin, whereas PAR-2 or PAR-4 agonist peptides were without effect. Furthermore, a small chemical inhibitor of PAR-1 (RWJ 58259) and a palmitoylated PAR-1-blocking peptide inhibited plasmin-induced migration of $\alpha 9$ -CHO cells. These results suggest that plasmin induces migration by kringle-mediated binding to $\alpha 9\beta 1$ and simultaneous proteolytic activation of PAR-1 [10]. It is likely that other integrins that bind to plasmin may exert similar effects on plasmin signaling.

We propose a model (Figure 1) in which (1) upon plasminogen activation, integrin-binding site in plasmin is exposed. Note that plasminogen does not bind to integrins $\alpha v\beta 3$ or $\alpha 9\beta 1$. (2) Once activated, plasmin is able to bind to integrins on the cell surface through the kringle domains (since integrin-binding sites are exposed) and proteolytically activates PAR-1, which induces intracellular signaling. Plasmin is concentrated to the cell surface through integrin binding, and this process is probably critical since plasmin has much lower affinity to PAR-1 than thrombin. Angiostatin,

in contrast, binds to integrins, but does not activate PAR-1. Angiostatin is expected to suppress plasmin action by competing with plasmin for binding to integrins.

It has been reported that integrins $\alpha M\beta 2$ [11], $\alpha D\beta 2$ [12], and $\alpha 5\beta 1$ [13] bind to plasminogen, while we did not detect binding of $\alpha v\beta 3$ or $\alpha 9\beta 1$ to plasminogen. One possibility is that integrins $\alpha M\beta 2$, $\alpha D\beta 2$, and $\alpha 5\beta 1$ recognize plasminogen in the ways different from those of $\alpha v\beta 3$ or $\alpha 9\beta 1$. Another possibility is that integrin-binding sites in plasminogen (perhaps kringle domains) are exposed in partially denatured plasminogen. Supporting the second possibility we observed that freshly prepared plasminogen did not significantly bind to $\alpha v\beta 3$, but plasminogen binding to $\alpha v\beta 3$ appeared to increase as plasminogen preparations aged (data not shown). This issue should be clarified in future studies.

In conclusion, the kringle domains in plasmin are involved in direct integrin binding, in addition to binding to the C-terminal Lysine residues of many proteins, and playing a role in inducing intracellular signals through proteolytic activation of PAR-1. The kringle-integrin interaction may enhance the cell surface concentration of plasmin, or directly induce intracellular signals through outside-in integrin signaling. Interestingly, plasminogen does not interact with integrins $\alpha v\beta 3$ or $\alpha 9\beta 1$ (possibly the integrin-binding sites are cryptic in plasminogen) (Figure 1) Based on our results on the plasmin kringle-integrin interaction, we hypothesized that the kringle domains of other serine proteases may interact with integrins and the interaction may play a role in their functions. Consistent with this idea, kringle domains from other proteins such as tissue-type plasminogen activator (tPA) [14] and apolipoprotein [15] have been reported to interact with integrins. This suggests that kringle-integrin interaction is a common mechanism in kringle-containing proteins.

2. uPA Kringle-Integrin Interaction

uPA is a highly restricted serine protease that converts the zymogen plasminogen to active plasmin. uPA binds with high affinity to a cell-surface uPA receptor (uPAR) that has been identified in many cell types. uPAR is a glycosylphosphatidylinositol- (GPI-) anchored 35–55 kDa glycoprotein. This system mediates pericellular proteolysis of extracellular matrix proteins including fibrin degradation (fibrinolysis) and plays an important role in cancer, inflammation, and immune responses [16–19]. The single chain form of uPA has three independently folded domains: the growth factor-like domain (GFD) (residue 1–46), kringle (residue 47–135) domain, and serine protease domain (residue 159–411). Enzymatic digestion of single chain-uPA yields an amino terminal fragment (ATF), which consists of the GFD and kringle domains, and the low molecular weight fragment (LMW-uPA), which consists of the serine protease domain. The uPAR-binding site of uPA is located in the GFD domain [20]; this binding is stabilized by the kringle [21]. It has generally been accepted that uPA signaling involves its binding to uPAR through its GFD [22].

uPA binding to uPAR on the cell surface facilitates activation of plasminogen to plasmin in vitro by increasing the rate

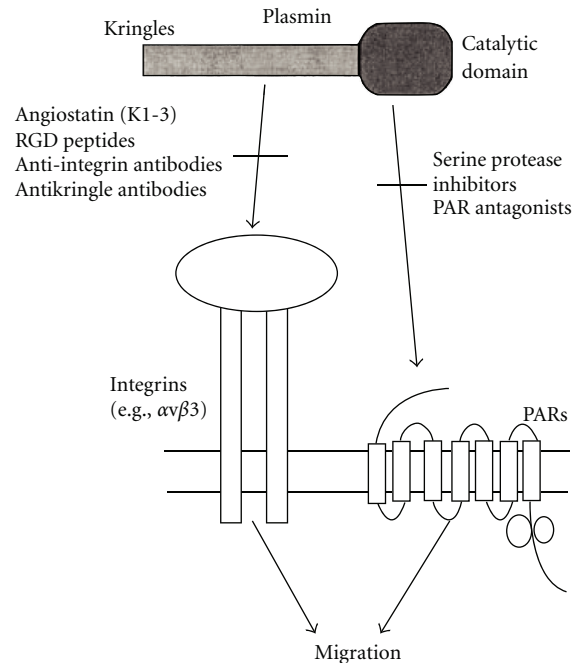


FIGURE 1: A model of plasmin-induced cell migration and the potential mechanism of angiostatin action. uPA activates plasminogen to plasmin pericellularly. Plasmin is accumulated on the cell surface by binding to integrins and stabilized. Free plasmin would be rapidly inactivated by circulating serine protease inhibitors (e.g., β 2-antiplasmin). The catalytic activity of plasmin on the cell surface is directly involved in signal transduction, possibly through activating G-protein coupled PARs. The binding of the kringle domain may not be directly involved in signaling through integrin pathways. Angiostatin effectively blocks plasmin-induced cell migration possibly by competing with plasmin for binding to integrins. Aprotinin, a serine protease inhibitor, also effectively blocks migration. It should be noted that other antiangiogenic agents, RGD-peptide and anti- α v β 3, are effective inhibitors of this process.

of pro-uPA activation by plasmin, by decreasing the apparent K_m of uPA to plasmin, and by increasing the K_{cat}/K_m of uPA to plasmin [23]. It is interesting that uPA-knockout mice do not have major thrombotic disorders [24]. This is probably because of the redundant fibrinolytic function by tPA. Indeed, combined uPA and tPA knockout mice show extensive thrombotic disorders very similar to those observed in plasminogen-knockout mice, but these are rarely detected in animals lacking uPA or tPA alone [25]. In contrast to uPA, studies performed in uPAR-knockout mice do not really support a major role of uPAR in fibrinolysis. Fibrin deposits are found within the livers of mice with a combined deficiency in uPAR and tPA, but not in uPAR-knockout mice, indicating a minor role for uPAR in plasminogen activation [25]. The extraordinarily mild consequences of combined uPAR and tPA deficiency raised the question of whether there are other receptors for uPA that might facilitate plasminogen activation [19, 25].

Besides plasminogen activation, uPA has been shown to induce the adhesion and chemotactic movement of myeloid cells [26, 27], to induce cell migration in human epithelial cells [28] and bovine endothelial cells [29], and to promote cell growth [30–32]. Notably these signaling functions of uPA do not require its proteolytic activity. Several studies suggest that uPA has additional, unidentified cell-surface receptor(s) other than uPAR that are involved in signaling events. For

example, blocking of uPA binding to uPAR using a monoclonal antibody or by depletion of cell surface uPAR with phosphatidylinositol-specific phospholipase C (PIPLC) did not inhibit uPA-induced mitogenic effects in smooth muscle cells [33]. uPA-induced mitogenic effects in melanoma cells are independent of high-affinity binding to uPAR, and this suggests the existence of a low-affinity binding site on this cell type based on the kinetic data [34]. The chemotactic action of uPA on smooth muscle cells depends on its kringle domain, and kinetic evidence indicates that these cells express a lower-affinity kringle receptor distinct from uPAR [35]. The isolated uPA kringle augments vascular smooth muscle cell constriction in vitro [36] and in vivo [37]. Taken together these observations all suggest that cells express uPA-binding proteins (other than uPAR) that mediate signaling from uPA.

We found that uPA binds specifically to integrin α v β 3 on CHO cells depleted of uPAR (Figure 2). The binding of uPA to α v β 3 required the uPA kringle domain (Figure 3). The isolated uPA kringle domain binds specifically to purified, recombinant soluble, and cell surface α v β 3, and other integrins (α 4 β 1 and α 9 β 1), and induces migration of CHO cells in an α v β 3-dependent manner. The binding of the uPA kringle to α v β 3 and uPA kringle-induced α v β 3-dependent cell migration is blocked by angiostatin. We studied whether the binding of uPA to integrin α v β 3 through the kringle

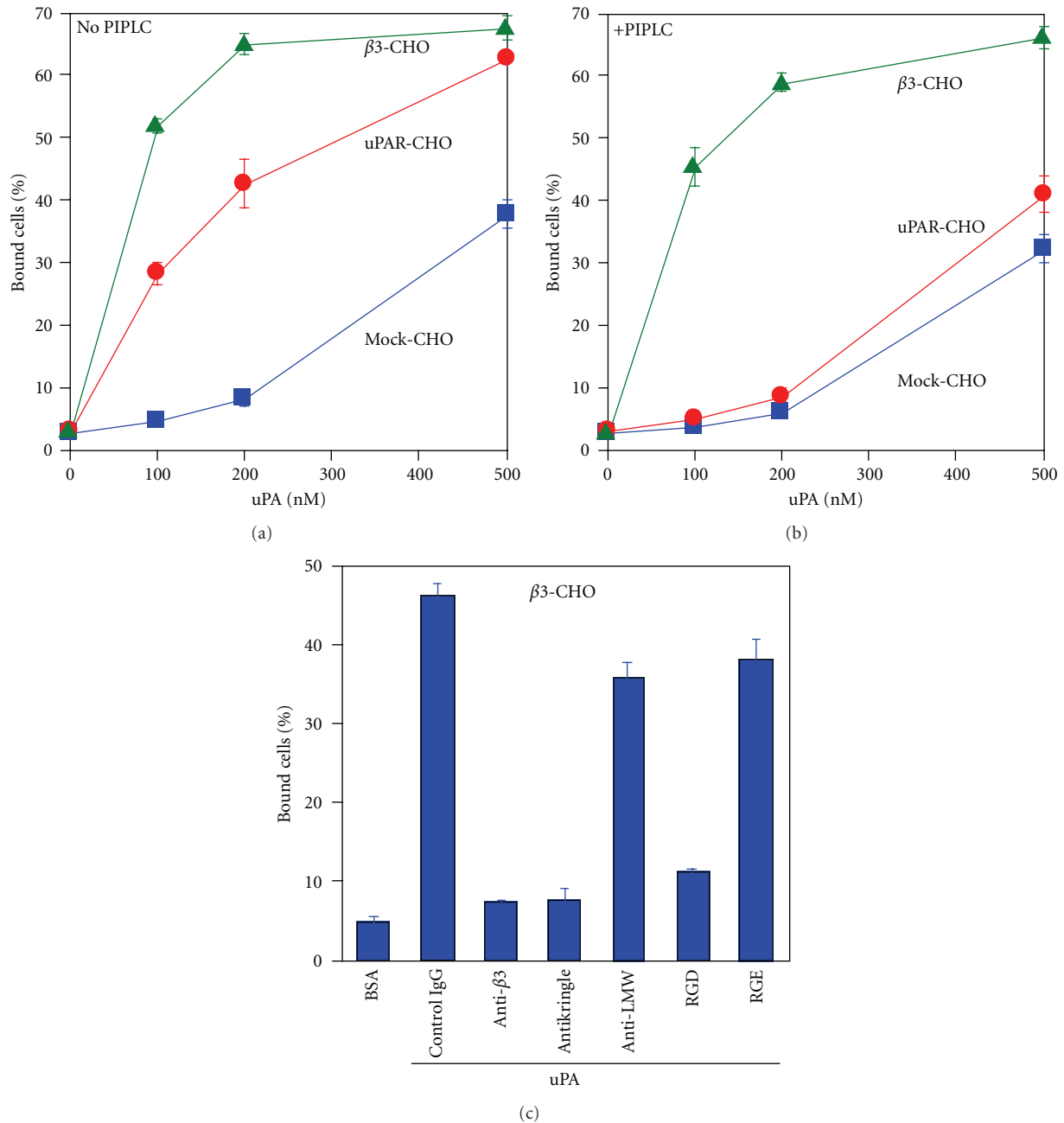


FIGURE 2: uPA binding to the cell surface in an integrin $\alpha v \beta 3$ -dependent and uPAR-independent manner [38]. (a) and (b) Depletion of uPAR from the cell surface blocked uPA binding to uPAR-CHO cells, but did not affect uPA binding to $\beta 3$ -CHO cells. To deplete GPI-linked uPAR on the cell surface, $\beta 3$ -CHO, uPAR-CHO, or control mock-transfected CHO cells were treated with PIPLC. The treatment removed more than 95% of human uPAR from uPAR-CHO cells as determined by flow cytometry with anti-uPAR mAb 3B10 (data not shown). uPA was immobilized to wells of 96-well microtiter plates at the indicated coating concentrations, and incubated with cells without (a) or with (b) pretreatment with PI-PLC. Bound cells were quantified. (c) uPA binding to $\beta 3$ -CHO cells is specific to $\alpha v \beta 3$ and the kringle domain. uPA (200 nM coating concentration) was immobilized to wells of 96-well microtiter plates and incubated with $\beta 3$ -CHO cells in the presence of mAb 16N7C2 (anti- $\beta 3$), Ab 963 (anti-kringle), mAb UNG-5 (anti-LMW-uPA), or RGD or RGE peptides (100 μ M).

domain plays a role in plasminogen activation. On CHO cell depleted of uPAR, uPA enhances plasminogen activation in a kringle and $\alpha v \beta 3$ -dependent manner (Figure 4). Endothelial cells bind to and migrate on uPA and uPA kringle in an $\alpha v \beta 3$ -dependent manner. These results suggest that uPA binding

to integrins through the kringle domain plays an important role in both plasminogen activation and uPA-induced intracellular signaling. The uPA kringle-integrin interaction may represent a novel therapeutic target for cancer, inflammation, and vascular remodeling [38].

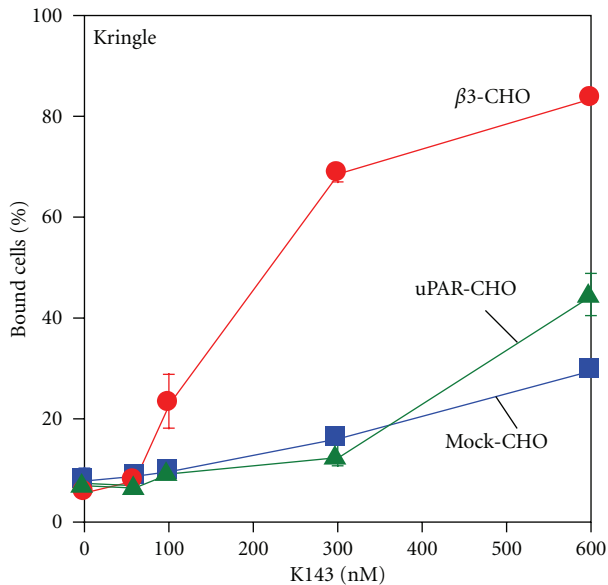


FIGURE 3: The kringle domain of uPA mediates binding to $\alpha\beta3$. The uPA kringle domain was immobilized onto wells of 96-well microtiter plates at the indicated coating concentrations and incubated with $\beta3$ -CHO, uPAR-CHO, or mock-CHO cells. The ability of the uPA fragments to support adhesion of these cells was determined [38].

We propose a model, in which the N-terminal GFD of uPA binds to uPAR and the kringle domain of uPA binds to integrins, leading to the uPAR-uPA-integrin ternary complex on the cell surface. It is likely that the ternary complex formation may be involved in uPA signaling and plasminogen activation. The isolated kringle or the isolated GFD domain may suppress uPA signaling or plasminogen activation by suppressing the process. Indeed isolated kringle domain or GFD have been shown to suppress tumorigenesis [39].

3. Another Example of the Role of $\alpha\beta3$ in uPA Signaling: uPA Kringle and Integrin $\alpha\beta3$ in Neutrophil Activation

It has been reported that antibody to integrin $\alpha\beta3$ and RGD peptide suppress the signaling action of uPA in neutrophils, although it is unclear if this include direct uPA- $\alpha\beta3$ interaction [40]. The study examined the ability of specific uPA domains to increase cytokine expression in murine and human neutrophils stimulated with lipopolysaccharides (LPS). Whereas the addition of intact uPA to neutrophils cultured with LPS increased mRNA and protein levels of interleukin-1 β , macrophage-inflammatory protein-2, and tumor necrosis factor α , deletion of the kringle domain from uPA resulted in loss of these potentiating effects. Addition of purified uPA kringle domain to LPS-stimulated neutrophils increased cytokine expression to a degree comparable with that produced by single-chain uPA. Inclusion of the RGD but not the RGE peptide to neutrophil cultures blocked uPA kringle-induced potentiation of proinflammatory responses,

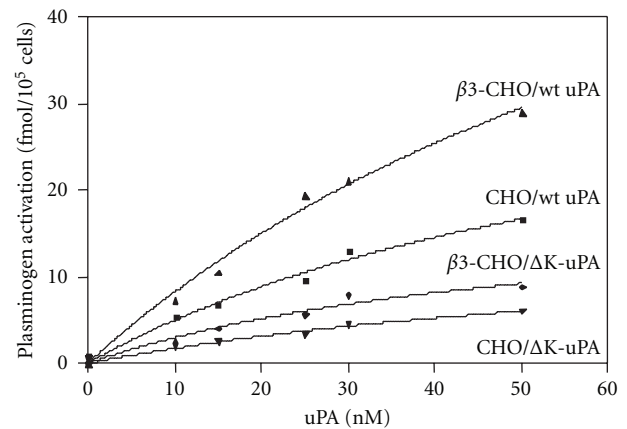


FIGURE 4: Integrin-dependent plasminogen activation on the cell surface. Parental CHO cells and $\beta3$ -CHO cells in wells of 96-well plates were treated with PIPLC to deplete uPAR, and incubated with wt or delta kringle (ΔK) uPA in the cold binding buffer for 1 h at 4°C. The cells were washed with the binding buffer, and plasminogen activation was determined using Glu-plasminogen and SpectrozymePL chromogenic substrate at 37°C. We found that $\beta3$ -CHO cells showed much higher ability to activate plasminogen in a manner dependent on the uPA added. Deletion of the kringle domain (with ΔK -uPA) markedly reduced the plasminogen activation on $\beta3$ -CHO, indicating that $\alpha\beta3$ and uPA-dependent plasminogen activation required the kringle domain of uPA. These results suggest that the binding of uPA kringle to integrin $\alpha\beta3$ induces plasminogen activation [38].

demonstrating that interactions between the kringle domain and integrins are involved. Antibodies to the α or $\beta3$ subunit or to $\alpha\beta3$ heterodimer prevented uPA kringle-induced enhancement of expression of proinflammatory cytokines and also of adhesion of neutrophils to the uPA kringle domain. These results demonstrate that the kringle domain of uPA, through interaction with $\alpha\beta3$ integrins, potentiates neutrophil activation.

4. A Docking Model of uPAR-uPA Kringle-Integrin Interaction

How does integrin $\alpha\beta3$ interact with uPA kringle? This has recently been predicted by docking simulation [41]. They modeled the interaction of uPA on two integrins, $\alpha IIB\beta3$ in the open configuration and $\alpha\beta3$ in the closed configuration. They found that multiple lowest energy solutions point to an interaction of the kringle domain of uPA at the boundary between α and β chains on the surface of the integrins. This region is not far away from peptides that have been previously shown to have a biological role in uPAR/integrins dependent signaling. They demonstrated that in silico docking experiments can be successfully carried out to identify the binding mode of the kringle domain of uPA on the scaffold of integrins in the open and closed conformation. Importantly they found that the binding mode is the same on different integrins and in both configurations. To get a molecular view of the system is a prerequisite to unravel the complex

TABLE 1: Amino acid residues involved in $\alpha\beta$ 3-uPA kringle interaction in the docking model. Amino acid residues at the binding interface (within the 6 Angstrom) were selected using Swiss pdb viewer (v. 4.02).

$\alpha\beta$	β 3	uPA kringle
Ala149, Asp150, Tyr178, Gln214, Ala215, Ile216, Asp218, Asp219, Arg248	Tyr122, Ser123, Met124, Lys125, Asp126, Asp127, Asp179, Met180, Lys181, Thr182, Arg214, Arg216, Asp217, Ala218, Asp251, Ala252, Lys253, Thr311, Glu312, Asn313, Val314, Asn316, Val332, Leu333, Ser334, Met335, Asp336, Ser337	Ser47, Lys48, Thr49, Tyr51, Glu52, Gly53, Asn54, Gly55, His56, Phe57, Tyr58 Arg59, Tyr84, Asp90, Leu92, Gln93, Leu94, Asn104, Pro105, Asp106, Asn107, Arg108, Arg109, Arg110, Glu125

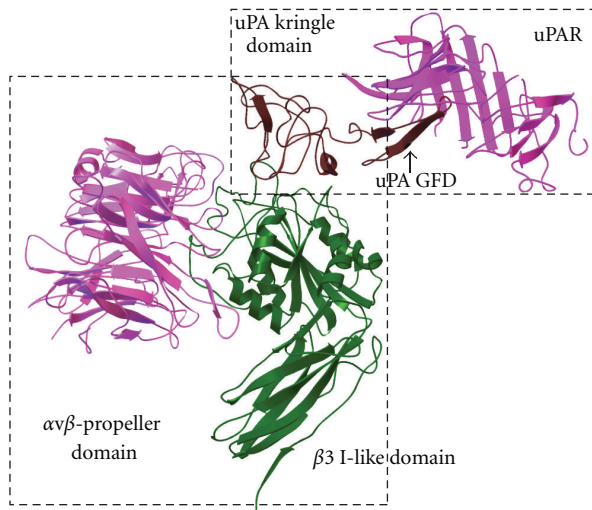


FIGURE 5: A model of integrin, uPA kringle, and uPAR complex. We performed docking simulation of the interaction between uPA kringle (PDB code 2URK) and integrin $\alpha\beta$ 3 (PDB code 1L5G) using Autodock3. The simulation predicted the poses in which uPA kringle interacts with $\alpha\beta$ 3. The uPA kringle-integrin complex was superposed with the ATF-uPAR complex (PDB code 2I9B).

protein-protein interactions underlying uPA/uPAR/integrin mediated cell motility, adhesion, and proliferation, and to design rational in vitro experiments.

However, in their paper which amino acid residues in uPA kringle are involved in integrin interaction is unclear. Thus, we presented our model here (Figure 5). We performed docking simulation of the interaction between uPA kringle (PDB code 2URK) and integrin $\alpha\beta$ 3 (PDB code 1L5G) using Autodock3. The simulation predicted the poses in which uPA kringle interacts with $\alpha\beta$ 3 (docking energy -22.3 kcal/mol). The amino acid residues involved in the interaction are shown in Table 1. The uPA kringle-binding site in $\alpha\beta$ 3 appears to be common to other known $\alpha\beta$ 3 ligands. The uPA kringle-integrin complex was superposed with the ATF-uPAR complex (PDB code 2I9B). Our model predicts that integrin $\alpha\beta$ 3 and uPAR can bind to ATF (GFD and kringle) simultaneously without steric hindrance. Obviously, it would be important to identify amino acid residues in uPA kringle that are critical for integrin binding by site-directed mutagenesis. In future studies, using uPA kringles that cannot bind to integrins or uPAR, it would be important to study the role of uPA kringle-integrin interaction in the proinflammatory action of uPA and to establish the role of uPAR in this process.

5. uPAR-Integrin Interaction

Previous studies suggest that uPAR directly binds to integrins [42–44]. How can our hypothesis explain this interaction? Our preliminary docking simulation studies of interaction between uPAR and integrin $\alpha\beta$ 3 did not detect high-affinity $\alpha\beta$ 3 binding sites in uPAR (not shown). In contrast the docking simulation of interaction between uPA kringle and $\alpha\beta$ 3 predicted high affinity binding of $\alpha\beta$ 3 to uPA kringle (as shown above). Since uPA binds to uPAR at high-affinity through GFD of uPA, one possibility is that previous studies detected interactions between the uPA-uPAR complex and integrins, in which integrins bind indirectly to uPAR through uPA kringle, but not those between uPAR and integrins. uPA is widely expressed in different cell types and tissues. This hypothesis should be rigorously tested in future studies.

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